

**Marked-up Version of Amended Specification and Claims
Pursuant to 37 C.F.R. §§ 1.121(b)-(c)**

In the Specification:

On page 1, after the title, the following paragraph has been inserted:

Priority Claim

This is a § 371 U.S. national stage of PCT/US00/25465, filed September 15, 2000, which was published in English under PCT Article 21(2), and claims the benefit of U.S. Application No. 60/154,601, filed September 17, 1999.

In the Abstract:

The attached abstract has been added to the end of the specification.

In the Claims:

The claims have been amended as follows:

1. (Amended) A **computer-implemented** method for counting nucleic acid probe signals in a region of interest in a biological specimen, the method comprising:

in a computer system, automatically counting a number of test signals from a test probe;

in the computer system, automatically counting a number of reference signals from a reference probe; and

in the computer system, determining a ratio of the **automatically**-counted test signals from the test probe to the **automatically**-counted reference signals from the reference probe, wherein the region of interest comprises multiple cells.

2. The method of claim 1, wherein the reference probe is a polynucleotide that hybridizes to a centromere, and the number of reference signals from the reference probe approximates a nucleus count in the biological specimen.

3. The method of claim 1, wherein the reference probe recognizes a target on a same chromosome as the test probe.

4. The method of claim 1, wherein the test probe is a polynucleotide that hybridizes to a target sequence in a gene, and the reference probe is a polynucleotide that hybridizes to a reference sequence.
5. The method of claim 3, wherein the reference probe recognizes a centromere of the same chromosome on which the gene of interest is contained.
6. The method of claim 1, further comprising obtaining successive images of the region of interest to distinguish overlapping signals in the biological specimen.
7. The method of claim 6, wherein the successive images are optical sections of the region of interest.
8. The method of claim 7, wherein the optical sections are at different depths of the biological specimen.
9. The method of claim 8, wherein the successive images are transformed into digital representations in which contiguous signal segments in successive optical sections are combined into a single signal in a particular optical section in which a strongest signal segment is located.
10. The method of claim 6, wherein different successive images are obtained for the test probe signals and the reference probe signals, and a quantity of test probe signals and reference probe signals are determined.
11. The method of claim 6, wherein successive images are obtained which show distinguishable test probe signals and reference probe signals, and a quantity of the test probe signals and reference probe signals are determined.

12. The method of claim 6, wherein the successive images are obtained by confocal microscopy.

13. The method of claim 1, wherein the ratio of signals is determined without reference to boundaries of a cell nucleus.

14. The method of claim 1, wherein the ratio of signals is determined without reference to the boundaries of a cell.

15. (Amended) [A method of counting] **The method of claim 1 wherein the probe signals are** visible signals from probes used with in situ hybridization of a biological [samples] **sample**, the method **further** comprising:
obtaining a plurality of images at different levels of the biological sample; **and**
constructing a three-dimensional image indicating discrete signals at different levels of the three-dimensional image; **and**
wherein automatically counting comprises counting **computer-identified discrete signals out of** the discrete signals at different levels of the three-dimensional image.

16. The method of claim 15, wherein the three-dimensional image is constructed by determining a location of a signal segment in the different levels of the biological sample, combining overlapping signal segments in contiguous levels into a single spot signal, and separating signal segments in non-contiguous levels into different spots.

17. The method of claim 16, wherein the location of signal segments is determined by the presence of an increase in brightness intensity that indicates an increase of signal as compared to a background signal.

18. The method of claim 17, wherein the probes display fluorescent signals, and the increase in brightness intensity is associated with an increase in fluorescence compared to the background signal.

19. The method of claim 15, wherein the signals comprise test signals from a test probe and reference signals from a reference probe.

20. The method of claim 19, wherein the test probe recognizes a gene of interest, and the reference probe recognizes a chromosomal locus having an expected quantity in the biological specimen.

21. The method of claim 20, further comprising determining a ratio between the test signals and the reference signals.

22. The method of claim 21, further comprising determining:

- (a) whether there is an increase in an expected ratio between the test signal and the reference signal, indicating an amplification of the gene of interest; or
- (b) whether there is a decrease in the expected ratio between the test signal and the reference signal, indicating relative loss of the gene of interest.

23. The method of claim 19, wherein the test probe is selected from the group consisting of probes that recognize genes implicated or suspected in the development or progression of a tumor.

24. **(Amended)** The method of claim 15, wherein the biological [samples are] sample is in a microarray.

25. The method of claim 24, wherein the microarray comprises a tissue microarray.

26. The method of claim 25, wherein the tissue microarray comprises tissue samples of a same tissue type taken from a plurality of donor specimens.

27. The method of claim 15, wherein the plurality of images consists of between eight and thirty two images at different levels of the biological sample.

28. The method of claim 15, further comprising:
avoiding counting discrete signals having intensities exceeding a threshold intensity.
29. The method of claim 15, further comprising:
avoiding counting discrete signals having a combined intensity and area exceeding a threshold value.
30. The method of claim 15, further comprising:
avoiding counting discrete signals related to autofluorescent material.
31. The method of claim 15, further comprising:
depicting a two-dimensional image representing the three-dimensional image for consideration by a user.
32. The method of claim 31, further comprising:
emphasizing discrete signals related to autofluorescent material in the two-dimensional image.
33. The method of claim 15, further comprising:
identifying a set of one or more discrete signals as a cluster; and
counting the cluster as a number of discrete signals greater than the number of discrete signals in the set.
34. The method of claim 33 wherein the cluster is counted as a number of discrete signals indicated by applying a mapping to the number of discrete signals in the set.
35. The method of claim 33 wherein the cluster is counted as a number of discrete signals indicated by a function calibrated via manual counting of spots in a plurality of images.
36. The method of claim 33 wherein the cluster is counted as a number of discrete signals indicated by a gain factor applied to the number of discrete signals in the set.

58. A computer-readable medium comprising computer-executable instructions for performing the following:

- within a stack of image slices generated from a plurality of confocal microscopic observations of a FISH experiment as a plurality of depths along a z-axis, identifying possible fluorescent image components;

- projecting the possible fluorescent image components within the image slices onto a projection image;

- discarding insignificant contiguous possible fluorescent image components in the slices;

- for each contiguous region in the projection image, grouping regions of possible fluorescent image components associated with the contiguous region in the projection image into spot candidates;

- applying a filter to the spot candidates; and

- counting the remaining spot candidates as spots.

59. The computer-readable medium of claim 58 wherein insignificant contiguous possible fluorescent image components are determined by comparing a size of a contiguous possible fluorescent image component with a threshold.

60. A computer-generated user interface for presenting results of microscopic observation of biological tissue subjected to a FISH experiment, the user interface comprising:

- a scatter plot of sets of image components designated as spot candidates for the FISH experiment;

- wherein the scatter plot comprises points indicating a size and intensity of spot candidates.

61. The computer-generated user interface of claim 60 wherein at least one point is operable to receive a user interface activation to navigate to a user interface display of information for a spot candidate associated with the point.

62. The computer-generated user interface of claim 60 wherein at least one point is operable to receive a user interface activation to navigate to a user interface display of a three-dimensional depiction of a spot candidate associated with the point.

63. The computer-generated user interface of claim 60 further comprising:
a display image depicting a view of the tissue subjected to the FISH experiment and a depiction of least one candidate spot thereon;
wherein the depiction of the candidate spot is operable to receive a user interface activation to designate the spot as a minimal intensity spot; and
wherein candidate spots designated as minimal intensity spots are visually emphasized when presenting the scatter plot.